Immunological Analysis of Cell-Associated Antigens of Bacillus anthracis

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By using electrophoretic immuno-transblots, EITB (Western Blots), we compared sera from Hartley guinea pigs vaccinated with a veterinary live-spore anthrax vaccine to those vaccinated with the human anthrax vaccine, consisting of aluminum hydroxide-adsorbed culture proteins of Bacillus anthracis strain V770-NP-1R. Sera from animals vaccinated with the spore vaccine recognized two major B. anthracis vegetative-cell-associated proteins not recognized by sera from animals receiving the human vaccine.

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These proteins, termed extractable antigens 1 (EA1) and 2 (EA2), have molecular masses of 91 and 62 kilodaltons, respectively. The EA1 protein appeared to be coded by chromosomal DNA, whereas the EA2 protein was only detected in strains possessing the pXO1 toxin plasmid. Both of the EA proteins were serologically distinct from the three anthrax toxin components, as determined by monoclonal antibody to protective antigen, edema factor, and lethal factor, and specific antisera to the EA proteins.

ABSTRACT

By using electrophoretic immuno-transblots, EITB (Western blots), we compared sera from Hartley guinea pigs vaccinated with a veterinary live-spore anthrax vaccine to those vaccinated with the human anthrax vaccine, consisting of aluminum hydroxide-adsorbed culture proteins of Bacillus anthracis strain V770-NP-1R. Sera from animals vaccinated with the spore vaccine recognized two major B. anthracis vegetative-cell-associated proteins not recognized by sera from animals receiving the human vaccine. These proteins, termed extractable antigens 1 (EA1) and 2 (EA2), have molecular masses of 91 and 62 kilodaltons, respectively. The EA1 protein appeared to be coded by chromosomal DNA, whereas the EA2 protein was only detected in strains possessing the pXO1 toxin plasmid. Both of the EA proteins were serologically distinct from the three anthrax toxin components, as determined by monoclonal antibody to protective antigen, edema factor, and lethal factor, and specific antisera to the EA proteins.

Since the introduction of the live, attenuated culture vaccine by Pasteur, Chamberland, and Roux, in 1881 (3), vaccination against anthrax has been practiced throughout much of the world. Pasteur prepared the vaccine by growing Bacillus anthracis at elevated temperatures (i.e., 42 to 43°C) to produce attenuated cultures. Recently, it was determined that growth at these temperatures induces the loss of a 110 megadalton plasmid, pXO1, which codes for the anthrax toxin (17) comprised of protective antigen (PA), edema factor (EF) and lethal factor (LF) (15, 22, 23, 26). Since both the toxin and the poly-D-glutamate capsule, the latter coded for by the 60 megadalton plasmid, pXO2 (7, 28), are required for virulence, bacilli lacking either or both plasmids are avirulent.

Based on these findings, it has been proposed that Pasteur's attenuation process heat cured the majority of the bacilli in culture of their pXO1 plasmid and that the sublethal numbers of toxigenic bacilli which remained, provided protection (4, 9). Although generally effective, improperly prepared Pasteur-type vaccine lots occasionally produced disease in vaccinated animals (8), probably due to excessive numbers of toxigenic virulent bacilli remaining in the cultures used as vaccines.

In 1939, Sterne isolated a nonencapsulated-toxigenic variant ($pXO1^+$, $pXO2^-$), which was found to be safe and efficacious as a live spore vaccine against anthrax (24, 25). The Sterne vaccine rapidly replaced the Pasteur vaccine and has since been used extensively as a veterinary vaccine in livestock (8).

In contrast, the commercial product licensed for human use in the United States, supplied by the Michigan Department of Public Health (MDPH), consists of aluminum-hydroxide adsorbed culture filtrates of the nonencapsulated, toxigenic strain V770-NP-1R (2, 8, 20). The predominant component of the MDPH vaccine is PA.

Animals vaccinated with live Sterne spore vaccines are protected to a much greater degree against anthrax than those vaccinated with human vaccine or aluminum hydroxide-adsorbed purified PA (10). It is clear from these and other studies (12, 16) that vaccination with live spore vaccines provides not only a greater level of protection but also prolonged immunity.

In light of the above observations, we questioned whether there are proteins other than PA that are recognized serologically by serum from animals vaccinated with a live spore vaccine but not when vaccinated with the human vaccine. Identification of such proteins may explain the differences in protection with the two vaccines and may also identify de novo vaccine candidates for future evaluation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacillus anthracis strains were obtained from the U.S. Army Medical Research Institute of Infectious Diseases culture collection, Fort Detrick, Md. Descriptions of parental strains have been reported (16). Strains cured of their pX01 toxin plasmid, designated with the prefix "Δ", have been described previously (9). Frozen spore stock suspensions were revived on 5% sheep blood agar by incubating at 37°C for 18 to 20 h. Growth from blood agar cultures was inoculated into R-medium (21), buffered with 50 mM Tris-HCl, pH 7.5. The cultures, 500 ml in 2-liter sealed screw-top flasks, were incubated with shaking at 100 rpm, 37°C, for 18 to 20 h.

Cell extraction. Cells were harvested by centrifugation at 10^4 x g, for 15 min and washed in 100 ml of R-medium. The cells were suspended to 0.1 g wet cell wt. per ml in sodium dodecyl sulfate (SDS) extraction buffer (11) consisting of 5 mM Tris-HCl, 1% (wt/vol) SDS, 5 mM 2-mercaptoethanol (2-ME), pH 9.8. The suspension was heated to 70°C for 30 min, cooled to ambient temperatures, and centrifuged as described above. The supernatant, containing extracted proteins, was filter sterilized through a 0.22 μm, low protein-binding, cellulose acetate filter (Millipore, Bedford, Mass.).

Culture supernatants were filtered, placed in dialysis tubing (3,500 molecular weight cut off), concentrated at 4°C in polyethylene glycol, and reconstituted with 0.1 volumes of extraction buffer. The dialysis bags were re-sealed and boiled

for 1 min to release proteins which may have become bound to the tubing. The samples were transfered to a polypropylene vial, boiled an additional 4 min, and then stored at -20°C.

SDS-Polyacrylamide gel electrophoresis. To assure saturation of proteins with SDS, the cell extracts and concentrated culture fluids were mixed with 5X sample buffer to give a final concentration of 1% (wt/vol) SDS, 10% (vol/vol) glycerol, and 10 mM 2-ME and placed in a boiling water bath for 5 min. Samples were electrophoresed on SDS-PAGE gels (14) containing 10% (wt/vol) acrylamide (acrylamide:bis-acrylamide ratio = 37.5:1), pH 8.8 with 5% acrylamide stacking gel (pH 6.8).

Electrophoretic immunotransblot (EITB). Following SDS-PAGE, the proteins were electrophoretically transblotted onto nitrocellulose, Nc (27), in transblot buffer (20 mM Tris, 150 mM glycine, 20% methanol, 0.1% SDS; (pH 8.5 before the addition of methanol and SDS). After electrophoresis for 16 to 18 h at 150 mA, the 14- by 12-cm Nc sheet was washed once in Buffer A (0.5% (wt/vol) gelatin, 10 mM HEPES buffer, 100 mM NaCl, pH 7.5) and incubated in 25 ml quench buffer (0.5% gelatin, 30 mM Na citrate, 5% (wt/vol) non-fat powdered milk, 10 mM HEPES, 100 mM NaCl, pH 7.5) for 30 min at 37°C. The Nc was then washed twice in Buffer A and the sheet cut into strips, approximately 2.5-mm wide. Each strip was incubated in 5 ml of antiserum or monoclonal antibody (MAb) empirically diluted in Buffer A for 2 h at 37°C with gentle agitation by using a 25 well incubation tray (BioRad, Richmond, Calif.) that had been modified by

dividing each well with acrylic blocks to yield 50 wells. Each strip was washed 4 times with 10 ml of Buffer A, allowing each wash to soak for 3 min. For detection of mouse MAb, the strips were incubated for 1 h in rabbit anti-mouse IgG, IgA, and IgM (Calbiochem, La Jolla, Calif.) diluted 1:600 in Buffer B (10 mM HEPES, 100 mM NaCl, 3% (wt/vol) BSA (bovine serum albumin), pH 7.5) at 37°C, followed by four washes with Buffer A. Detection of the rabbit antibody or guinea pig antibody was accomplished by incubating the strips in horseradish peroxidase-protein-A conjugate (supplied by Dr. Stephen Leppla, USAMRIID), diluted 1:5000 in Buffer B for 30 min at ambient temperatures. The strips were then washed in Buffer C (5 mM HEPES, 100 mM NaCl, 0.05% NP-40, pH 7.5) for 5 min, followed by two 5-min washes in Buffer D (10 mM HEPES, 100 mM NaCl, pH 7.5). The conjugate-antibody complexes were detected using the chromogenic substrate [4 ml of tetramethylbenzidine (5 mg/ml in methanol); 16 ml of 1% dioctylsodium-sulfosuccinate (Sigma Chemical Co., St. Louis, Mo.); 1 ml of 1 M HEPES, pH 7.5; 59 ml distilled water (dH_2O); and 20 μl 3% H_2O_2]. The reaction was terminated at 10 min by washing the Nc strips three times in Buffer E (10 mM HEPES, 0.2% dioctylsodium-sulfosuccinate, 5% methanol, pH 7.5). Photographs of developed strips were made by using Polaroid film, Type 55 (OPELCO, Washington, DC).

Molecular mass determination. Molecular mass protein standards (Bio-Rad, Richmond, Calif.) included ovalbumin (45 kilodaltons), bovine serum albumin (66.2 Kdal), phosphorylase B (92.5 Kdal), β-galactosidase (116.5 Kdal), and myosin (200 Kdal). The standards were treated with 5X sample buffer as described above, electrophoresed in lanes adjacent to those containing cell extracts, transblotted, and the Nc stained with AuroDye (Janssen, Piscataway, N.J.) The electrophoretic mobilities of the standards were calculated, plotted, and the molecular mass of the unknown proteins interpolated.

Vaccinations. Unless indicated otherwise, female Hartley guinea pigs (Charles River Laboratories, Wilmington, Mass.) received three, bi-weekly, 0.5 ml intramuscular (i.m.) injections of the Michigan Department of Public Health (MDPH) human vaccine. Animals vaccinated with the Sterne veterinary vaccine (Anvax, Wellcome Animal Research Laboratories) were given three bi-weekly i.m. injections, consisting of 2×10^6, 4×10^5, and 5×10^6 spores, respectively. After i.m. administration of 30 mg ketamine per kg (Vetalar, Parke Davis, Morris Plains, N.J.) and 6 mg of xylazine per kg (Rompun, Haver-Lockhardt, Shawnee, Kan.), the animals were bled by intracardiac puncture using a 23 gauge needle. Sera were diluted in glycerol (1:1) prior to freezing at $-20^{\circ}C$.

Sera to Nc bound EA proteins. Protein bands on Nc sheets were identified by performing EITB analyses on strips cut from the center and sides of each sheet. Upon realignment of the stained

strips with the unstained center sections, the identified protein bands of interest were cut out and the dried strips solubilized in a minimal amount of dimethyl sulfoxide (13). The solubilized, Nc-bound proteins were mixed with an equal volume of Freund's complete adjuvant and 0.2 ml was injected subcutaneously in the hind quarters of each test animal. The guinea pigs received an additional injection the second week by using Freund's incomplete adjuvant, and a third injection on week 4 without adjuvant. Animals were bled as described above.

Antibody titer determinations. Quantitation of antibody titers to PA in MDPH- and Anvax-vaccinated guinea pigs was accomplished using enzyme linked immunosorbent assay (ELISA). Purified PA was coated on 96-well, polystyrene, microtiter plates (Linbro) by incubating 100 µl of antigen (1 µg/ ml, 50 mM Na borate, pH 9) in the wells for 2 h at 37°C. Unreacted binding sites were blocked with quench buffer (10 mM Na phosphate, 0.85% NaCl, 0.5% gelatin, 5% powdered milk, pH 7.5) for 30 min at ambient temperature. Wells were washed 4X with phosphate-buffered saline (PBS) + 0.05% Tween-20 (PBST). Antisera were titrated in PBS + gelatin, incubated 2 h at 37°C, and the wells washed four times with PBST. Protein A/horseradish peroxidase conjugate, diluted 1:5000 in PBST with 5% heat-inactivated fetal calf serum, was added (100 µl per ml) and plates were incubated for 30 min at 37°C. The wells were washed four times with PBST. Freshly prepared 2,2'azino-bis(3 ethylbenzthiazoline-sulfonic acid (ABTS) (Sigma) substrate (100 µl per well) was added

and incubated at ambient temperature for 20 min. The reaction was stopped with 50 µl of 10% SDS. The concentration of ABTS was 1 mg/ml in 0.1 M citrate buffer, pH 4.0. Hydrogen peroxide was added to 0.003% just prior to use. Substrate and SDS were added to a blank well on all plates for background subtraction. Control plates, without antigen, were treated identically as the test plates, and their absorbance values subtracted from those of the test plates to correct for nonspecific binding of antibody. The ELISA plates were read 405 nm in a Bio-Tek EL308 Microplate reader (Bio-Tek Instruments, Inc., Burlington, Vt.). Titers were taken as being the last dilution with an absorbance $\geq$ 0.1.

RESULTS

Comparison of sera from MDPH- and Anvax-vaccinated animals. Whole cell extracts and 10X concentrated culture supernatant fluids were electrophoresed and transblotted to Nc. The Nc was cut into strips and incubated with sera, diluted 1:100 in Buffer A, washed and stained as described. As shown in Fig. 1 and 2, sera obtained from Anvax vaccinees recognized a 91 Kdal-protein, in Sterne cell extracts, referred to as extractable antigen 1 or EA1 (Fig. 3). In contrast, this protein was not recognized by sera from the MDPH vaccinated animals. The same observations were made with cell extracts from the virulent Vollum-1B strain (Fig. 2) and B. anthracis strains Ames, New Hampshire, Colorado, and V770-NP-1R (data not shown). Inspection of both Fig. 1 and 2 reveals that PA was also present in the cell extract in significant quantities. This observation was based not only on the relative intensity of the EITB bands on Nc strips, but also on Kodavue- and Coomassie Blue R-250-stained SDS-PAGE gels.

Both groups of animals produced high antibody titers to PA as determined by ELISA, Table 1 [the identifying numbers for the sera shown in the table correspond to the sera numbers in Fig. 1 and 2]. As shown in Table 1, the animals vaccinated with the MDPH human vaccine had 2- to 4-fold higher titers to PA than did the animals vaccinated with Anvax. This is consistent with the EITB analyses in that the relative intensity of PA staining by the sera from Sterne strain (Anvax)-vaccinated animals was somewhat diminished as compared to the sera from MDPH-vaccinated animals.

Except where indicated, the EITB analyses of sera shown in Fig. 1 and 2 were obtained from animals 2 weeks after vaccination. Based on the relative intensity of the bands, it appeared initially that EA1 was the principle cell-associated antigen recognized by the Anvax-vaccinated animals, rather than PA. However, in guinea pigs that were vaccinated with the MDPH human vaccine, PA was the primary antigen recognized, as was also the case with antisera from MDPH-vaccinated humans (Fig. 2, strips 1 and 2).

As shown in Fig. 1B and 2B, EA1 does not appear to be a major component of the culture supernatant, but is clearly present, as seen on strips 7 and 8 in Fig. 2B. Preliminary studies with antisera to EA1 are consistent with the observations that EA1 was released by vegetative cells into the culture supernatant.

The PA protein appears to give rise to several breakdown products, including those identified by the letters B and C in Fig. 1B, 2 and 4, which may be a result of protease activity. These breakdown products reacted with MAb to PA, while another major band, labeled with the letter A, was weakly stained by MAb to LF and EF (Fig. 4). Sera from guinea pigs that had been boosted 12 weeks after the initial vaccination regimen revealed an additional extractable antigen, termed EA2 (62 Kdal) (Fig. 4). This protein was not well recognized by sera collected 2 weeks after the initial three-vaccination series (Fig. 2A). The EA2 protein was extracted only from strains carrying the pXO1

toxin plasmid, whereas EA1 was extracted from both plasmid-carrying and -cured strains (Table 2).

MAb and specific antiserum. Mouse MAb was used to identify the three anthrax toxin components to distinguish them from the EA proteins. The MAb preparations were supplied by Stephen Little, USAMRIID, as ascities fluid and were used at an empirically determined dilution of 1:50. As shown in Fig. 3, Sterne cell-extracted antigens were stained with antisera from two guinea pigs vaccinated with Anvax (strips 1 and 2) and MDPH (strips 3 and 4). As shown in strips 5 and 6, MAb to EF, diluted 1:5 and 1:50, respectively, stained a protein slightly larger than PA, but distinct from EA1 and EA2. Strips 7 and 8 were stained with two different MAb to LF (diluted 1:50) and 9 and 10 with two different MAb to PA (diluted 1:50). In all cases, the toxin proteins were distinguishable from the EA proteins.

Additional evidence that the EA proteins are distinct was provided by EITB analyses of antisera to the EA proteins. Antiserum to EA1, extracted from a Sterne strain cured of the pXO1 toxin plasmid, stained both EA1 and a second protein which appears slightly above EA1 on EITB strips (Fig 5). However, antiserum to EA2, extracted from the parental Sterne strain containing the pXO1 plasmid, stained EA2 and weakly stained LF (data not shown). It was subsequently found that Nc strips, which were solubilized in dimethyl sulfoxide for vaccination into

guinea pigs, also contained a breakdown product of LF, which migrates slightly ahead of EA2 on SDS-PAGE gels (indicated by the letter A in Fig. 1B, 2, and 4).

DISCUSSION

Since the discovery of the three anthrax toxin components (1, 5, 22, 29), substantial emphasis has been placed on the release of these proteins by B. anthracis, both in vivo and in vitro. Much of this emphasis has been on PA and its central role in the activity, or transport, of EF and LF in host target cells (18, 6). With PA playing such a central role, many have held that antibody induced to this protein would protect the vaccinee by neutralizing toxin activity. Indeed, this may be partially accurate; however, one cannot discount the reports that antibody titers to PA do not correlate well with protection and that protection in animals vaccinated with live spore vaccines is far better and more prolonged than in those vaccinated with chemical vaccines (i.e., MDPH, adsorbed PA) (10, 16). Little and Knudson (16) reported that, although titers to PA were higher in animals vaccinated with the MDPH human vaccine as opposed to those vaccinated with Anvax, only the latter were completely protected against "vaccine-resistant" B. anthracis strains. The sera used in Fig. 1 and 2 and in Table 1 were from a similar study in which the Anvax-vaccinated guinea pigs were afforded significantly higher and more prolonged protection, yet had lower antibody titers to PA (manuscript in preparation).

Although protection following vaccination with either of the EA proteins has not been demonstrated, consideration must now be given to the potential role of these proteins and/or other cell constituents as immunogens. The observation that animals

vaccinated with the veterinary live-spore vaccine produce antibody to PA, EA1, and EA2, whereas animals vaccinated with the MDPH vaccine produce antibody to PA but not EA1 or EA2, suggests such a role. However, alternative explanations for the differences in protection afforded by the two vaccines must be explored. For example, it may be that PA is the primary immunogen in both vaccines, but its presentation to the host's immune system by live-spore vaccines elicits an immune response (i.e., cell-mediated immunity) not obtained with aluminum hydroxide adsorbed vaccines. Alternatively, crucial epitopes on the PA molecule, which are expressed on PA released by live vaccines, may be either destroyed or masked when bound to aluminum hydroxide.

Presently, the role(s) of the EA proteins is not understood. The EA1 protein does not appear to be plasmid mediated and data to date indicate that it may not be protective. In experiments not presented in this report, guinea pigs were vaccinated with EA1 bound to nitrocellulose. Although all the animals developed antibody titers to EA1 (Fig. 5), none of the animals survived either aerosol or i.m. lethal challenge with Vollum 1B spores; whereas, all control animals vaccinated with Anvax vaccine survived. These results agree well with the observations that live vaccines composed of B. anthracis strains lacking the pXO1 plasmid, but still producing EA1, are not protective (9). Since protection with live vaccines has only been accomplished with strains which carry the pXO1 plasmid, one may postulate that

macromolecules coded for by the pXO1 plasmid are required for protection. To date, those molecules coded for by the pXO2 capsule plasmid do not appear to serve as immunogens, as evidenced by protection with Sterne strain vaccines ($pXO1^+$, $pXO2^-$). Because EA2 has been detected only in strains which carry the pXO1 plasmid, its potential role(s) in infection and immunogenesis are being investigated by our group. Thus far, preliminary challenge studies in animals vaccinated with Nc-bound EA2 have been hampered by the fact that the animals also develop antibody to LF due to contamination of EA2 preparations with a LF breakdown product. This LF fragment (approximately 60 Kdal) migrates very close to EA2 on SDS-PAGE, thereby making it difficult to cut strips from EITB transblots that contain only the EA2 protein. It is clear that other approaches to this problem must be explored.

The PA protein was demonstrated to be extractable from whole cells by Puziss and Howard in 1963 (19). To our knowledge, the extraction of EF and LF from intact anthrax bacilli has never been reported. The concentrations of EF and LF in culture fluids are only 1 to 2 μg/ml (15). If these proteins are retained intracellularly under alkaline growth conditions, as suggested by Puziss and Howard for PA, then perhaps their isolation from whole cells may provide increased yields.

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TABLE 1. Antibody titers to Protective Antigen for sera from MDPH- and Anvax-vaccinated guinea pigs.

Strip #[a]			
Fig. 1	Fig. 2	Vaccine	Titer[b]
1	16	MDPH	16,384
2	15	MDPH	16,384
3	14	MDPH	32,768
4	13	MDPH	32,768
5	12	MDPH	32,768
6	11	Supernatant[c]	ND[d]
7	10	Anvax	ND
8	9	Anvax	2,048
9	8	Anvax	2,048
10	7	Anvax	8,192
11	6	Anvax	16,382
12	5	Saline	0
13	4	Saline	0
14	3	Saline	0

a The strips are those identified in Fig. 1 and 2.

b Reciprocal titer as determined by ELISA.

c Animals vaccinated with culture supernatant fluid from Sterne strain cultures.

d Not determined

TABLE 2. Association of EA proteins with plasmids pXO1 and pXO2 among *B. anthracis* strains.

	Plasmid			
Strain	pXO1	pXO2	EA1	EA2
Vollum 1B	+	+	+	+
New Hampshire	+	+	+	+
Ames	+	+	+	+
Sterne	+	-	+	+
V770-NP-1R	+	-	+	+
Δ Ames	-	+	+	-
Δ New Hampshire	-	+	+	-
Δ Sterne	-	-	+	-

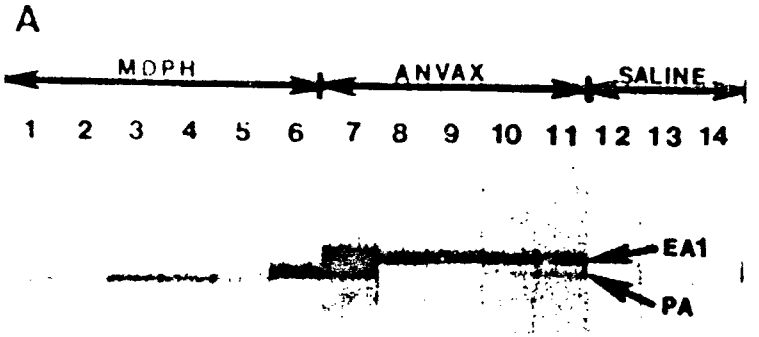
FIG. 1. (A) Comparison of sera from MDPH- and Anvax-vaccinated guinea pigs by EITB with SDS-extracted proteins from Sterne strain vegetative cells. (B) Comparison of sera by using culture supernatant proteins from the Sterne cells in Fig. 1-A. The strips were incubated with sera (diluted 1:100) obtained from animals 2 weeks after being vaccinated with three biweekly injections of either MDPH human vaccine (strips 1 to 6) or Anvax live spore vaccine (strips 7 to 11). Strips 12 to 14 were incubated with sera from control animals injected with saline.

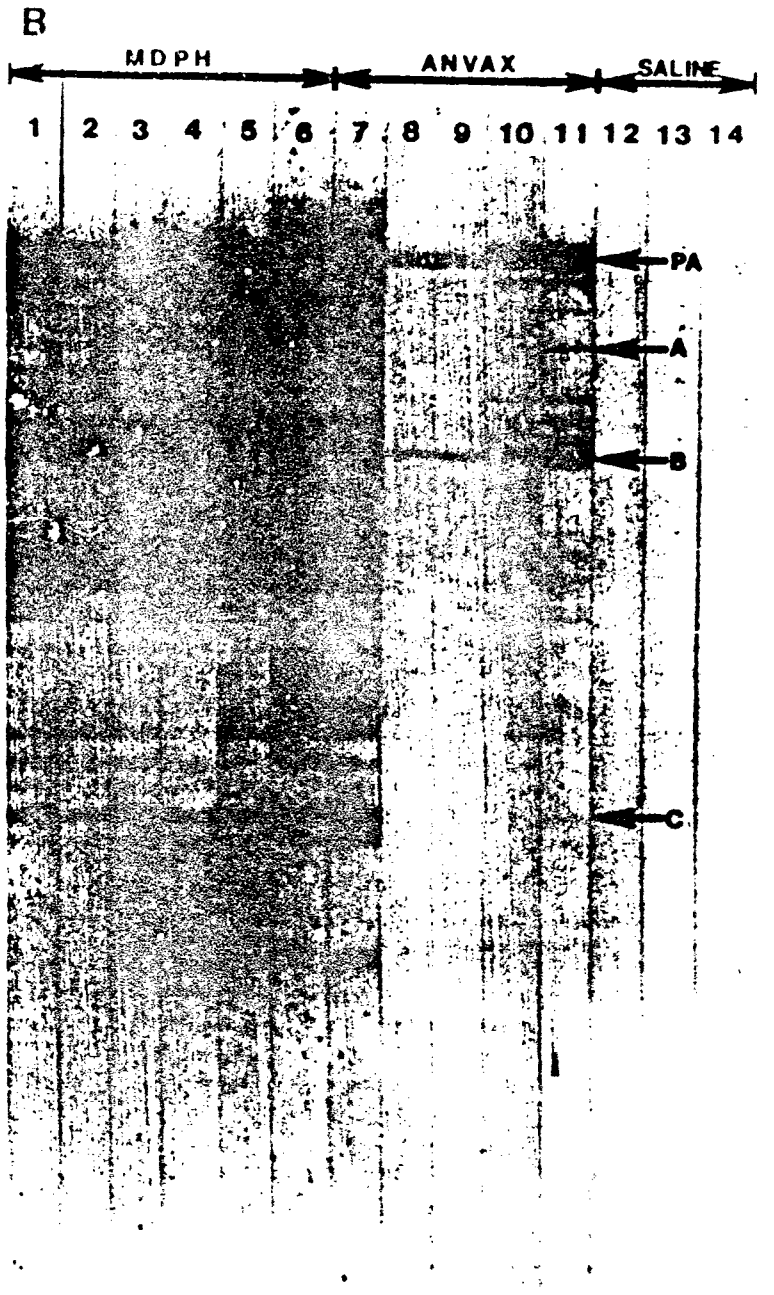
FIG. 2. (A) Comparison of sera from MDPH- and Anvax-vaccinated guinea pigs by EITB on SDS-extracted proteins from Vollum 1-B strain vegetative cells. (B) Comparison of sera by using culture supernatant proteins from the Vollum 1-B cells in Fig. 2-A. The protein preparations were treated as described for FIG. 1. The sera were from animals vaccinated with either the Anvax live spore vaccine (strips 6 to 10) or the MDPH human vaccine (strips 11 to 16). Strips 3 to 5 were incubated with sera from control animals that had been given saline injections. Strips 1 and 2 were reacted with sera from two human subjects that had been vaccinated with 0.5 ml MDPH vaccine (three biweekly injections with booster vaccinations at 6 and 12 months).

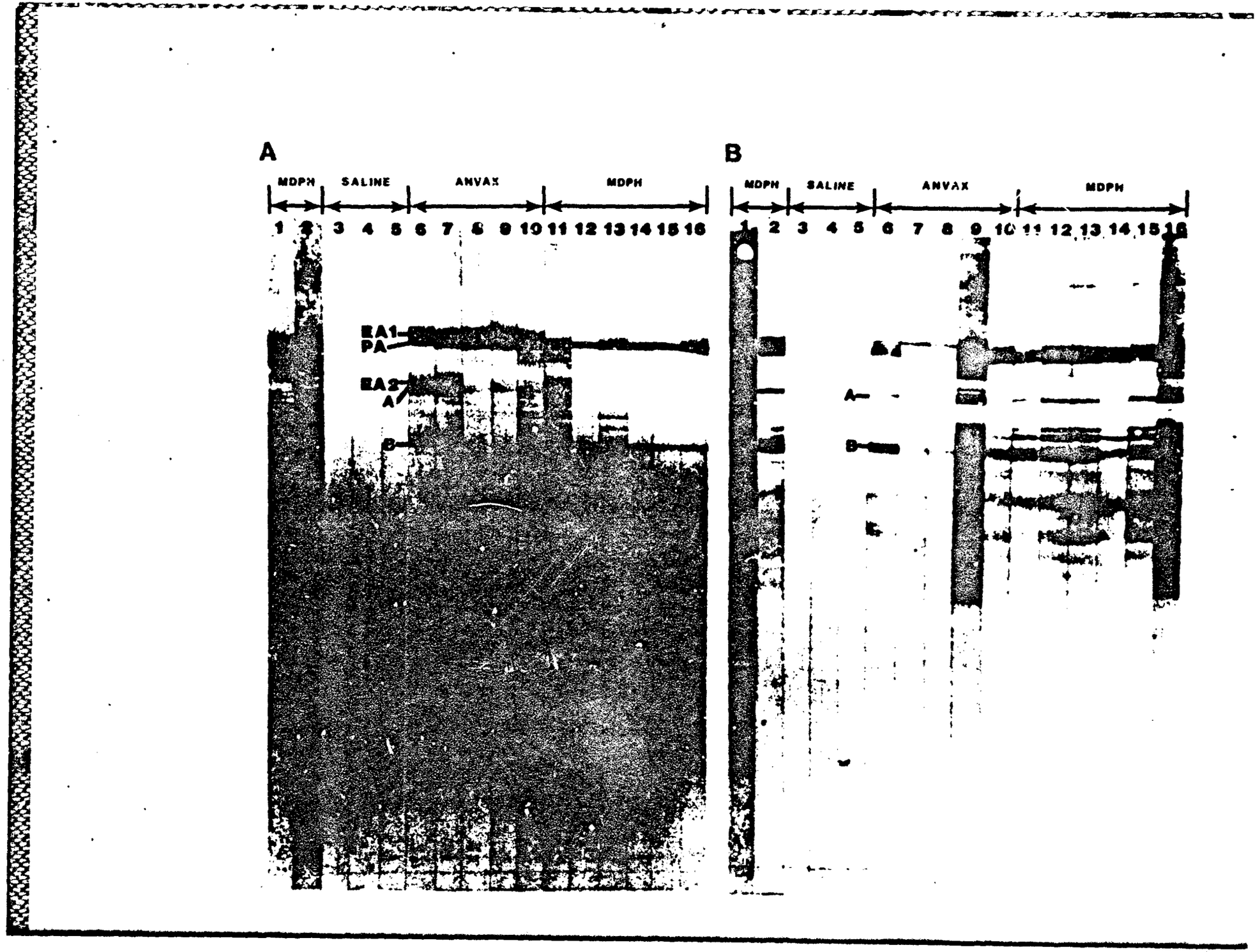
FIG. 3. Approximations of the molecular mass of EA1, EA2, and PA are indicated. Molecular mass protein standards were: (I) ovalbumin, 45 Kdal; (II) bovine serum albumin, 66.2 Kdal; (III) phosphorylase B, 92.5 Kdal; (IV) β-galactosidase, 116.5 Kdal; and (V) myosin. 200 Kdal.

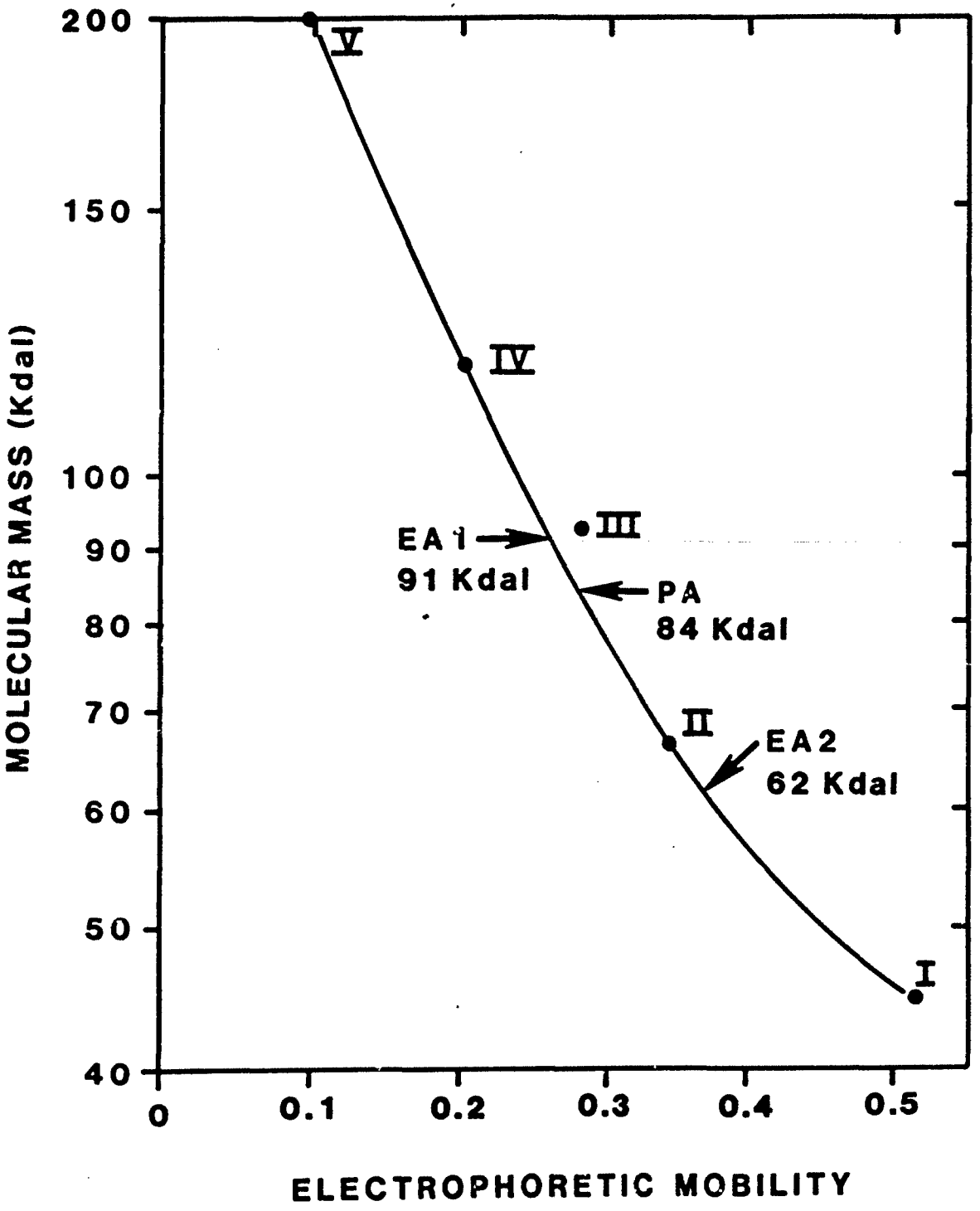
FIG. 4. EITB analysis of Sterne cell-extracted proteins with sera from guinea pigs vaccinated with Anvax (strips 1 and 2) and MDPH (strips 3 and 4). Strips 5 and 6 were reacted with MAb to EF (MAb# EF-III 9F5-1-1, tissue culture fluid) diluted 1:5 and 1:50, respectively. Two different MAb ascities fluid preparations specific for LF (MAb# LF-III 2B2-1-2 and LF-II 3E3-3-1) were used stain strips 7 and 8, respectively. MAb preparations to PA (MAb# PA-I 3B6-1-1 and PA-I 2D3-3-1) were used to stain strips 9 and 10, respectively. The ascities fluids were diluted 1:50.

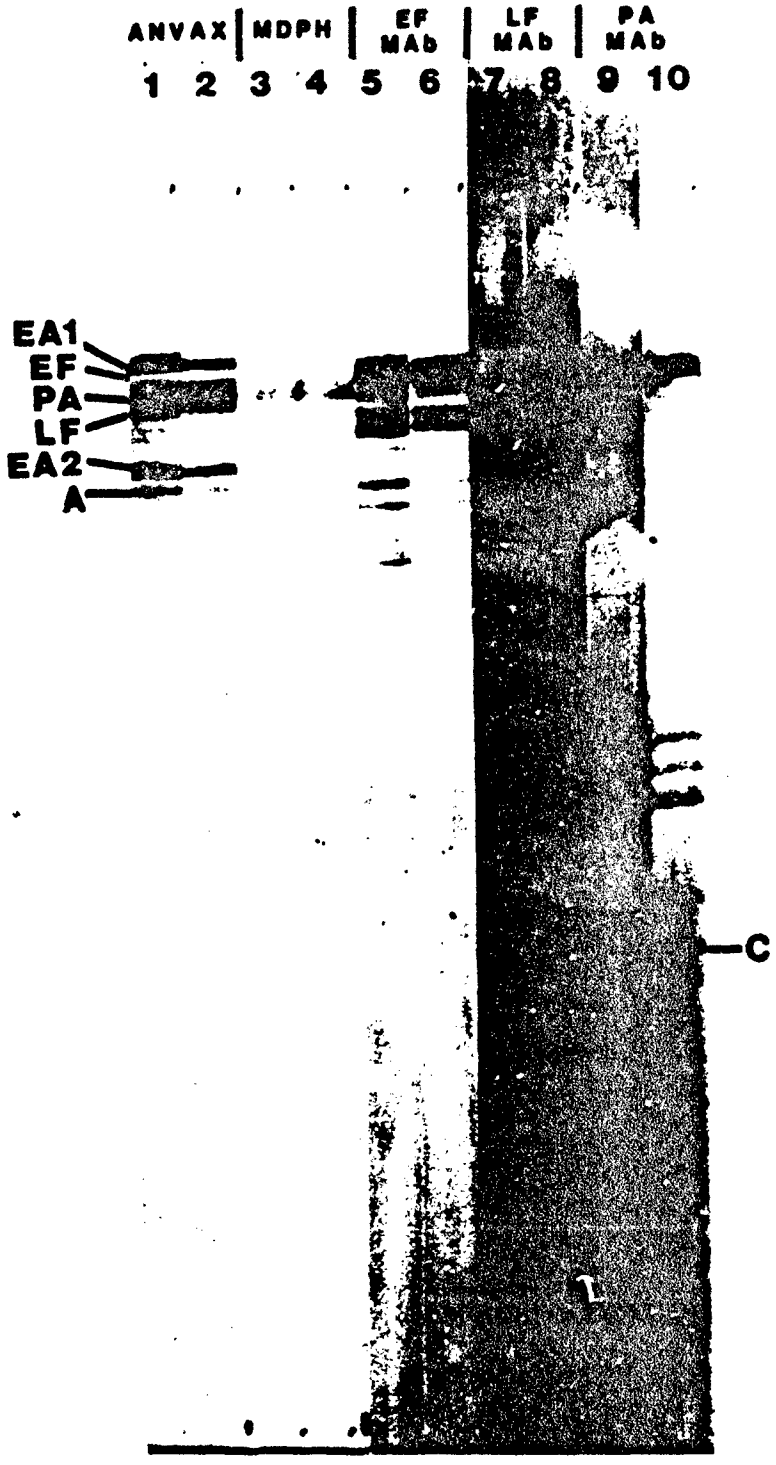
FIG. 5. EITB analysis of Sterne cell extracted proteins with sera from guinea pigs vaccinated with EA1 bound to Nc (strips 1 through 5), Anvax (strips 6 and 7), and MDPH (strips 8 and 9).

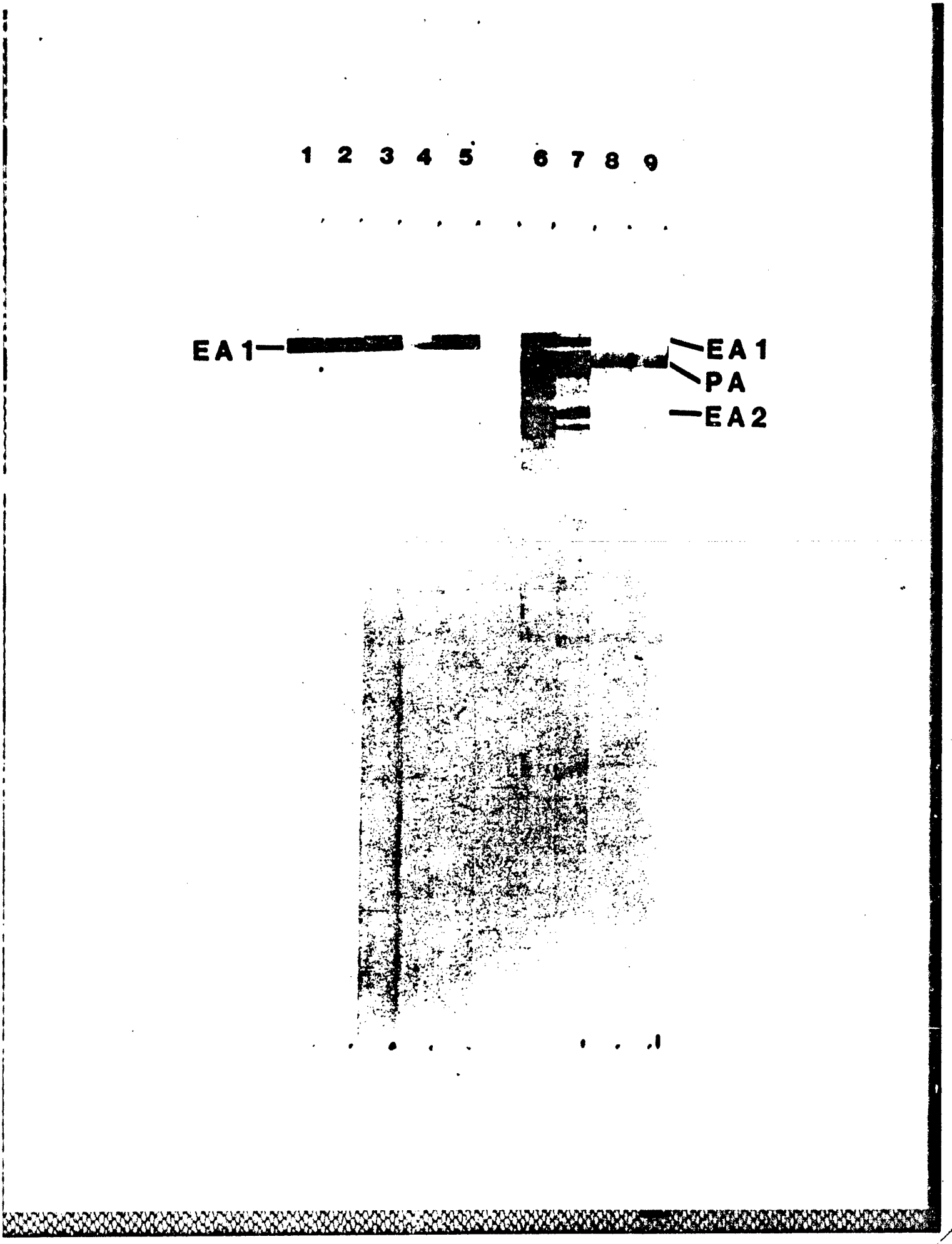

A
MDPH
ANVAX
SALINE
1 2 3 4 5 6 7 8 9 10 11 12 13 14
EA1
PA


B
MDPH
ANVAX
SALINE
1 2 3 4 5 6 7 8 9 10 11 12 13 14
PA
A
B
C


A
MDPH
SALINE
ANVAX
MDPH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
EA1
PA
EA2
A
B
B
MDPH
SALINE
ANVAX
MDPH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
A
B


200
150
100
90
80
70
60
50
40
MOLECULAR MASS (Kdal)
V
IV
III
II
I
EA 1
91 Kdal
PA
84 Kdal
EA 2
62 Kdal
0
0.1
0.2
0.3
0.4
0.5
ELECTROPHORETIC MOBILITY


ANVAX
MDPH
EF MAb
LF MAb
PA MAb
1 2 3 4 5 6 7 8 9 10
EA1
EF
PA
LF
EA2
A
C


1 2 3 4 5 6 7 8 9
EA1
EA1
PA
EA2

Immunological Analysis of Cell-Associated Antigens of *Bacillus anthracis*

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Running Title: *B. anthracis* antigens.

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ABSTRACT

By using electrophoretic immuno-transblots, EITB (Western blots), we compared sera from Hartley guinea pigs vaccinated with a veterinary live-spore anthrax vaccine to those vaccinated with the human anthrax vaccine, consisting of aluminum hydroxide-adsorbed culture proteins of Bacillus anthracis strain V770-NP-1R. Sera from animals vaccinated with the spore vaccine recognized two major B. anthracis vegetative-cell-associated proteins not recognized by sera from animals receiving the human vaccine. These proteins, termed extractable antigens 1 (EA1) and 2 (EA2), have molecular masses of 91 and 62 kilodaltons, respectively. The EA1 protein appeared to be coded by chromosomal DNA, whereas the EA2 protein was only detected in strains possessing the pXO1 toxin plasmid. Both of the EA proteins were serologically distinct from the three anthrax toxin components, as determined by monoclonal antibody to protective antigen, edema factor, and lethal factor, and specific antisera to the EA proteins.

Since the introduction of the live, attenuated culture vaccine by Pasteur, Chamberland, and Roux, in 1881 (3), vaccination against anthrax has been practiced throughout much of the world. Pasteur prepared the vaccine by growing Bacillus anthracis at elevated temperatures (i.e., 42 to 43°C) to produce attenuated cultures. Recently, it was determined that growth at these temperatures induces the loss of a 110 megadalton plasmid, pXO1, which codes for the anthrax toxin (17) comprised of protective antigen (PA), edema factor (EF) and lethal factor (LF) (15, 22, 23, 26). Since both the toxin and the poly-D-glutamate capsule, the latter coded for by the 60 megadalton plasmid, pXO2 (7, 28), are required for virulence, bacilli lacking either or both plasmids are avirulent.

Based on these findings, it has been proposed that Pasteur's attenuation process heat cured the majority of the bacilli in culture of their pXO1 plasmid and that the sublethal numbers of toxigenic bacilli which remained, provided protection (4, 9). Although generally effective, improperly prepared Pasteur-type vaccine lots occasionally produced disease in vaccinated animals (8), probably due to excessive numbers of toxigenic virulent bacilli remaining in the cultures used as vaccines.

In 1939, Sterne isolated a nonencapsulated-toxigenic variant ($pXO1^+$, $pXO2^-$), which was found to be safe and efficacious as a live spore vaccine against anthrax (24, 25). The Sterne vaccine rapidly replaced the Pasteur vaccine and has since been used extensively as a veterinary vaccine in livestock (8).

In contrast, the commercial product licensed for human use in the United States, supplied by the Michigan Department of Public Health (MDPH), consists of aluminum-hydroxide adsorbed culture filtrates of the nonencapsulated, toxigenic strain V770-NP-1R (2, 8, 20). The predominant component of the MDPH vaccine is PA.

Animals vaccinated with live Sterne spore vaccines are protected to a much greater degree against anthrax than those vaccinated with human vaccine or aluminum hydroxide-adsorbed purified PA (10). It is clear from these and other studies (12, 16) that vaccination with live spore vaccines provides not only a greater level of protection but also prolonged immunity.

In light of the above observations, we questioned whether there are proteins other than PA that are recognized serologically by serum from animals vaccinated with a live spore vaccine but not when vaccinated with the human vaccine. Identification of such proteins may explain the differences in protection with the two vaccines and may also identify <u>de novo</u> vaccine candidates for future evaluation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacillus anthracis strains were obtained from the U.S. Army Medical Research Institute of Infectious Diseases culture collection, Fort Detrick, Md. Descriptions of parental strains have been reported (16). Strains cured of their pXO1 toxin plasmid, designated with the prefix "Δ", have been described previously (9). Frozen spore stock suspensions were revived on 5% sheep blood agar by incubating at 37°C for 18 to 20 h. Growth from blood agar cultures was inoculated into R-medium (21), buffered with 50 mM Tris-HCl, pH 7.5. The cultures, 500 ml in 2-liter sealed screw-top flasks, were incubated with shaking at 100 rpm, 37°C, for 18 to 20 h.

Cell extraction. Cells were harvested by centrifugation at 10^4 x g, for 15 min and washed in 100 ml of R-medium. The cells were suspended to 0.1 g wet cell wt. per ml in sodium dodecyl sulfate (SDS) extraction buffer (11) consisting of 5 mM Tris-HCl, 1% (wt/vol) SDS, 5 mM 2-mercaptoethanol (2-ME), pH 9.8. The suspension was heated to 70°C for 30 min, cooled to ambient temperatures, and centrifuged as described above. The supernatant, containing extracted proteins, was filter sterilized through a 0.22 μm, low protein-binding, cellulose acetate filter (Millipore, Bedford, Mass.).

Culture supernatants were filtered, placed in dialysis tubing (3,500 molecular weight cut off), concentrated at 4°C in polyethylene glycol, and reconstituted with 0.1 volumes of extraction buffer. The dialysis bags were re-sealed and boiled

for 1 min to release proteins which may have become bound to the tubing. The samples were transfered to a polypropylene vial, boiled an additional 4 min, and then stored at -20°C.

SDS-Polyacrylamide gel electrophoresis. To assure saturation of proteins with SDS, the cell extracts and concentrated culture fluids were mixed with 5X sample buffer to give a final concentration of 1% (wt/vol) SDS, 10% (vol/vol) glycerol, and 10 mM 2-ME and placed in a boiling water bath for 5 min. Samples were electrophoresed on SDS-PAGE gels (14) containing 10% (wt/vol) acrylamide (acrylamide:bis-acrylamide ratio = 37.5:1), pH 8.8 with 5% acrylamide stacking gel (pH 6.8).

Electrophoretic immunotransblot (EITB). Following SDS-PAGE, the proteins were electrophoretically transblotted onto nitrocellulose, Nc (27), in transblot buffer (20 mM Tris, 150 mM glycine, 20% methanol, 0.1% SDS; (pH 8.5 before the addition of methanol and SDS). After electrophoresis for 16 to 18 h at 150 mA, the 14- by 12-cm Nc sheet was washed once in Buffer A (0.5% (wt/vol) gelatin, 10 mM HEPES buffer, 100 mM NaCl, pH 7.5) and incubated in 25 ml quench buffer (0.5% gelatin, 30 mM Na citrate, 5% (wt/vol) non-fat powdered milk, 10 mM HEPES, 100 mM NaCl, pH 7.5) for 30 min at 37°C. The Nc was then washed twice in Buffer A and the sheet cut into strips, approximately 2.5-mm wide. Each strip was incubated in 5 ml of antiserum or monoclonal antibody (MAb) empirically diluted in Buffer A for 2 h at 37°C with gentle agitation by using a 25 well incubation tray (BioRad, Richmond, Calif.) that had been modified by

dividing each well with acrylic blocks to yield 50 wells. Each strip was washed 4 times with 10 ml of Buffer A, allowing each wash to soak for 3 min. For detection of mouse MAb, the strips were incubated for 1 h in rabbit anti-mouse IgG, IgA, and IgM (Calbiochem, La Jolla, Calif.) diluted 1:600 in Buffer B (10 mM HEPES, 100 mM NaCl, 3% (wt/vol) BSA (bovine serum albumin), pH 7.5) at 37°C, followed by four washes with Buffer A. Detection of the rabbit antibody or guinea pig antibody was accomplished by incubating the strips in horseradish peroxidase-protein-A conjugate (supplied by Dr. Stephen Leppla, USAMRIID), diluted 1:5000 in Buffer B for 30 min at ambient temperatures. The strips were then washed in Buffer C (5 mM HEPES, 100 mM NaCl, 0.05% NP-40, pH 7.5) for 5 min, followed by two 5-min washes in Buffer D (10 mM HEPES, 100 mM NaCl, pH 7.5). The conjugate-antibody complexes were detected using the chromogenic substrate [4 ml of tetramethylbenzidine (5 mg/ml in methanol); 16 ml of 1% dioctylsodium-sulfosuccinate (Sigma Chemical Co., St. Louis, Mo.); 1 ml of 1 M HEPES, pH 7.5; 59 ml distilled water (dH_2O); and 20 μl 3% H_2O_2]. The reaction was terminated at 10 min by washing the Nc strips three times in Buffer E (10 mM HEPES, 0.2% dioctylsodium-sulfosuccinate, 5% methanol, pH 7.5). Photographs of developed strips were made by using Polaroid film, Type 55 (OPELCO, Washington, DC).

Molecular mass determination. Molecular mass protein standards (Bio-Rad, Richmond, Calif.) included ovalbumin (45 kilodaltons), bovine serum albumin (66.2 Kdal), phosphorylase B (92.5 Kdal), β-galactosidase (116.5 Kdal), and myosin (200 Kdal). The standards were treated with 5X sample buffer as described above, electrophoresed in lanes adjacent to those containing cell extracts, transblotted, and the Nc stained with AuroDye (Janssen, Piscataway, N.J.) The electrophoretic mobilities of the standards were calculated, plotted, and the molecular mass of the unknown proteins interpolated.

Vaccinations. Unless indicated otherwise, female Hartley guinea pigs (Charles River Laboratories, Wilmington, Mass.) received three, bi-weekly, 0.5 ml intramuscular (i.m.) injections of the Michigan Department of Public Health (MDPH) human vaccine. Animals vaccinated with the Sterne veterinary vaccine (Anvax, Wellcome Animal Research Laboratories) were given three bi-weekly i.m. injections, consisting of 2×10^6, 4×10^6, and 5×10^6 spores, respectively. After i.m. administration of 30 mg ketamine per kg (Vetalar, Parke Davis, Morris Plains, N.J.) and 6 mg of xylazine per kg (Rompun, Haver-Lockhardt, Shawnee, Kan.), the animals were bled by intracardiac puncture using a 23 gauge needle. Sera were diluted in glycerol (1:1) prior to freezing at $-20^{\circ}C$.

Sera to Nc bound EA proteins. Protein bands on Nc sheets were identified by performing EITB analyses on strips cut from the center and sides of each sheet. Upon realignment of the stained

strips with the unstained center sections, the identified protein bands of interest were cut out and the dried strips solubilized in a minimal amount of dimethyl sulfoxide (13). The solubilized, Nc-bound proteins were mixed with an equal volume of Freund's complete adjuvant and 0.2 ml was injected subcutaneously in the hind quarters of each test animal. The guinea pigs received an additional injection the second week by using Freund's incomplete adjuvant, and a third injection on week 4 without adjuvant. Animals were bled as described above.

Antibody titer determinations. Quantitation of antibody titers to PA in MDPH- and Anvax-vaccinated guinea pigs was accomplished using enzyme linked immunosorbent assay (ELISA). Purified PA was coated on 96-well, polystyrene, microtiter plates (Linbro) by incubating 100 µl of antigen (1 µg/ ml, 50 mM Na borate, pH 9) in the wells for 2 h at 37°C. Unreacted binding sites were blocked with quench buffer (10 mM Na phosphate, 0.85% NaCl, 0.5% gelatin, 5% powdered milk, pH 7.5) for 30 min at ambient temperature. Wells were washed 4X with phosphate-buffered saline (PBS) + 0.05% Tween-20 (PBST). Antisera were titrated in PBS + gelatin, incubated 2 h at 37°C, and the wells washed four times with PBST. Protein A/horseradish peroxidase conjugate, diluted 1:5000 in PBST with 5% heat-inactivated fetal calf serum, was added (100 µl per ml) and plates were incubated for 30 min at 37°C. The wells were washed four times with PBST. Freshly prepared 2,2'azino-bis(3 ethylbenzthiazoline-sulfonic acid (ABTS) (Sigma) substrate (100 µl per well) was added

and incubated at ambient temperature for 20 min. The reaction was stopped with 50 µl of 10% SDS. The concentration of ABTS was 1 mg/ml in 0.1 M citrate buffer, pH 4.0. Hydrogen peroxide was added to 0.003% just prior to use. Substrate and SDS were added to a blank well on all plates for background subtraction. Control plates, without antigen, were treated identically as the test plates, and their absorbance values subtracted from those of the test plates to correct for nonspecific binding of antibody. The ELISA plates were read 405 nm in a Bio-Tek EL308 Microplate reader (Bio-Tek Instruments, Inc., Burlington, Vt.). Titers were taken as being the last dilution with an absorbance $\geq$ 0.1.

RESULTS

Comparison of sera from MDPH- and Anvax-vaccinated animals. Whole cell extracts and 10X concentrated culture supernatant fluids were electrophoresed and transblotted to Nc. The Nc was cut into strips and incubated with sera, diluted 1:100 in Buffer A, washed and stained as described. As shown in Fig. 1 and 2, sera obtained from Anvax vaccinees recognized a 91 Kdal-protein, in Sterne cell extracts, referred to as extractable antigen 1 or EA1 (Fig. 3). In contrast, this protein was not recognized by sera from the MDPH vaccinated animals. The same observations were made with cell extracts from the virulent Vollum-1B strain (Fig. 2) and B. anthracis strains Ames, New Hampshire, Colorado, and V770-NP-1R (data not shown). Inspection of both Fig. 1 and 2 reveals that PA was also present in the cell extract in significant quantities. This observation was based not only on the relative intensity of the EITB bands on Nc strips, but also on Kodavue- and Coomassie Blue R-250-stained SDS-PAGE gels.

Both groups of animals produced high antibody titers to PA as determined by ELISA, Table 1 [the identifying numbers for the sera shown in the table correspond to the sera numbers in Fig. 1 and 2]. As shown in Table 1, the animals vaccinated with the MDPH human vaccine had 2- to 4-fold higher titers to PA than did the animals vaccinated with Anvax. This is consistent with the EITB analyses in that the relative intensity of PA staining by the sera from Sterne strain (Anvax)-vaccinated animals was somewhat diminished as compared to the sera from MDPH-vaccinated animals.

Except where indicated, the EITB analyses of sera shown in Fig. 1 and 2 were obtained from animals 2 weeks after vaccination. Based on the relative intensity of the bands, it appeared initially that EA1 was the principle cell-associated antigen recognized by the Anvax-vaccinated animals, rather than PA. However, in guinea pigs that were vaccinated with the MDPH human vaccine, PA was the primary antigen recognized, as was also the case with antisera from MDPH-vaccinated humans (Fig. 2, strips 1 and 2).

As shown in Fig. 1B and 2B, EA1 does not appear to be a major component ... the culture supernatant, but is clearly present, as seer strips 7 and 8 in Fig. 2B. Preliminary studies with antisera to EA1 are consistent with the observations that EA1 was released by vegetative cells into the culture supernatant.

The PA protein appears to give rise to several breakdown products, including those identified by the letters B and C in Fig. 1B, 2 and 4, which may be a result of protease activity. These breakdown products reacted with MAb to PA, while another major band, labeled with the letter A, was weakly stained by MAb to LF and EF (Fig. 4). Sera from guinea pigs that had been boosted 12 weeks after the initial vaccination regimen revealed an additional extractable antigen, termed EA2 (62 Kdal) (Fig. 4). This protein was not well recognized by sera collected 2 weeks after the initial three-vaccination series (Fig. 2A). The EA2 protein was extracted only from strains carrying the pXO1

toxin plasmid, whereas EA1 was extracted from both plasmid-carrying and -cured strains (Table 2).

MAb and specific antiserum. Mouse MAb was used to identify the three anthrax toxin components to distinquish them from the EA proteins. The MAb preparations were supplied by Stephen Little, USAMRIID, as ascities fluid and were used at an empirically determined dilution of 1:50. As shown in Fig. 3, Sterne cell-extracted antigens were stained with antisera from two guinea pigs vaccinated with Anvax (strips 1 and 2) and MDPH (strips 3 and 4). As shown in strips 5 and 6, MAb to EF, diluted 1:5 and 1:50, respectively, stained a protein slightly larger than PA, but distinct from EA1 and EA2. Strips 7 and 8 were stained with two different MAb to LF (diluted 1:50) and 9 and 10 with two different MAb to PA (diluted 1:50). In all cases, the toxin proteins were distinguishable from the EA proteins.

Additional evidence that the EA proteins are distinct was provided by EITB analyses of antisera to the EA proteins. Antiserum to EA1, extracted from a Sterne strain cured of the pXO1 toxin plasmid, stained both EA1 and a second protein which appears slightly above EA1 on EITB strips (Fig 5). However, antiserum to EA2, extracted from the parental Sterne strain containing the pXO1 plasmid, stained EA2 and weakly stained LF (data not shown). It was subsequently found that Nc strips, which were solubilized in dimethyl sulfoxide for vaccination into

guinea pigs, also contained a breakdown product of LF, which migrates slightly ahead of EA2 on SDS-PAGE gels (indicated by the letter A in Fig. 1B, 2, and 4).

DISCUSSION

Since the discovery of the three anthrax toxin components (1, 5, 22, 29), substantial emphasis has been placed on the release of these proteins by *B. anthracis*, both in vivo and in vitro. Much of this emphasis has been on PA and its central role in the activity, or transport, of EF and LF in host target cells (18, 6). With PA playing such a central role, many have held that antibody induced to this protein would protect the vaccinee by neutralizing toxin activity. Indeed, this may be partially accurate; however, one cannot discount the reports that antibody titers to PA do not correlate well with protection and that protection in animals vaccinated with live spore vaccines is far better and more prolonged than in those vaccinated with chemical vaccines (i.e., MDPH, adsorbed PA) (10, 16). Little and Knudson (16) reported that, although titers to PA were higher in animals vaccinated with the MDPH human vaccine as opposed to those vaccinated with Anvax, only the latter were completely protected against "vaccine-resistant" *B. anthracis* strains. The sera used in Fig. 1 and 2 and in Table 1 were from a similar study in which the Anvax-vaccinated guinea pigs were afforded significantly higher and more prolonged protection, yet had lower antibody titers to PA (manuscript in preparation).

Although protection following vaccination with either of the EA proteins has not been demonstrated, consideration must now be given to the potential role of these proteins and/or other cell constituents as immunogens. The observation that animals

vaccinated with the veterinary live-spore vaccine produce antibody to PA, EA1, and EA2, whereas animals vaccinated with the MDPH vaccine produce antibody to PA but not EA1 or EA2, suggests such a role. However, alternative explanations for the differences in protection afforded by the two vaccines must be explored. For example, it may be that PA is the primary immunogen in both vaccines, but its presentation to the host's immune system by live-spore vaccines elicits an immune response (i.e., cell-mediated immunity) not obtained with aluminum hydroxide adsorbed vaccines. Alternatively, crucial epitopes on the PA molecule, which are expressed on PA released by live vaccines, may be either destroyed or masked when bound to aluminum hydroxide.

Presently, the role(s) of the EA proteins is not understood. The EA1 protein does not appear to be plasmid mediated and data to date indicate that it may not be protective. In experiments not presented in this report, guinea pigs were vaccinated with EA1 bound to nitrocellulose. Although all the animals developed antibody titers to EA1 (Fig. 5), none of the animals survived either aerosol or i.m. lethal challenge with Vollum 1B spores; whereas, all control animals vaccinated with Anvax vaccine survived. These results agree well with the observations that live vaccines composed of B. anthracis strains lacking the pXO1 plasmid, but still producing EA1, are not protective (9). Since protection with live vaccines has only been accomplished with strains which carry the pXO1 plasmid, one may postulate that

macromolecules coded for by the pXO1 plasmid are required for protection. To date, those molecules coded for by the pXO2 capsule plasmid do not appear to serve as immunogens, as evidenced by protection with Sterne strain vaccines ($pXO1^+$, $pXO2^-$). Because EA2 has been detected only in strains which carry the pXO1 plasmid, its potential role(s) in infection and immunogenesis are being investigated by our group. Thus far, preliminary challenge studies in animals vaccinated with Nc-bound EA2 have been hampered by the fact that the animals also develop antibody to LF due to contamination of EA2 preparations with a LF breakdown product. This LF fragment (approximately 60 Kdal) migrates very close to EA2 on SDS-PAGE, thereby making it difficult to cut strips from EITB transblots that contain only the EA2 protein. It is clear that other approaches to this problem must be explored.

The PA protein was demonstrated to be extractable from whole cells by Puziss and Howard in 1963 (19). To our knowledge, the extraction of EF and LF from intact anthrax bacilli has never been reported. The concentrations of EF and LF in culture fluids are only 1 to 2 μg/ml (15). If these proteins are retained intracellularly under alkaline growth conditions, as suggested by Puziss and Howard for PA, then perhaps their isolation from whole cells may provide increased yields.

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TABLE 1. Antibody titers to Protective Antigen for sera from MDPH- and Anvax-vaccinated guinea pigs.

Strip #[a]			
Fig. 1	Fig. 2	Vaccine	Titer[b]
1	16	MDPH	16,384
2	15	MDPH	16,384
3	14	MDPH	32,768
4	13	MDPH	32,768
5	12	MDPH	32,768
6	11	Supernatant[c]	ND[d]
7	10	Anvax	ND
8	9	Anvax	2,048
9	8	Anvax	2,048
10	7	Anvax	8,192
11	6	Anvax	16,382
12	5	Saline	0
13	4	Saline	0
14	3	Saline	0

[a] The strips are those identified in Fig. 1 and 2.

[b] Reciprocal titer as determined by ELISA.

[c] Animals vaccinated with culture supernatant fluid from Sterne strain cultures.

[d] Not determined

TABLE 2. Association of EA proteins with plasmids pXO1 and pXO2 among *B. anthracis* strains.

	Plasmid			
Strain	pXO1	pXO2	EA1	EA2
Vollum 1B	+	+	+	+
New Hampshire	+	+	+	+
Ames	+	+	+	+
Sterne	+	-	+	+
V770-NP-1R	+	-	+	+
Δ Ames	-	+	+	-
Δ New Hampshire	-	+	+	-
Δ Sterne	-	-	+	-

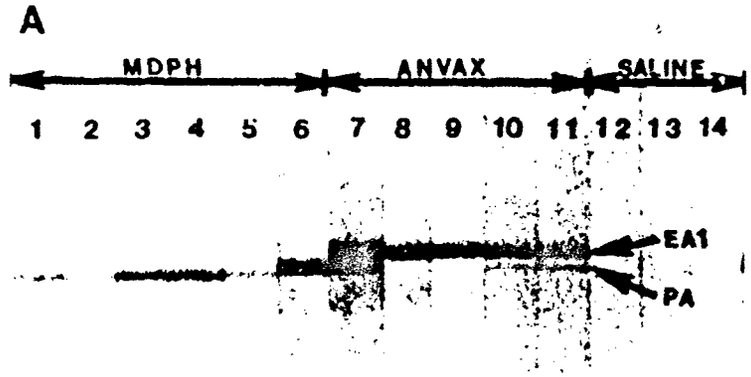
FIG. 1. (A) Comparison of sera from MDPH- and Anvax-vaccinated guinea pigs by EITB with SDS-extracted proteins from Sterne strain vegetative cells. (B) Comparison of sera by using culture supernatant proteins from the Sterne cells in Fig. 1-A. The strips were incubated with sera (diluted 1:100) obtained from animals 2 weeks after being vaccinated with three biweekly injections of either MDPH human vaccine (strips 1 to 6) or Anvax live spore vaccine (strips 7 to 11). Strips 12 to 14 were incubated with sera from control animals injected with saline.

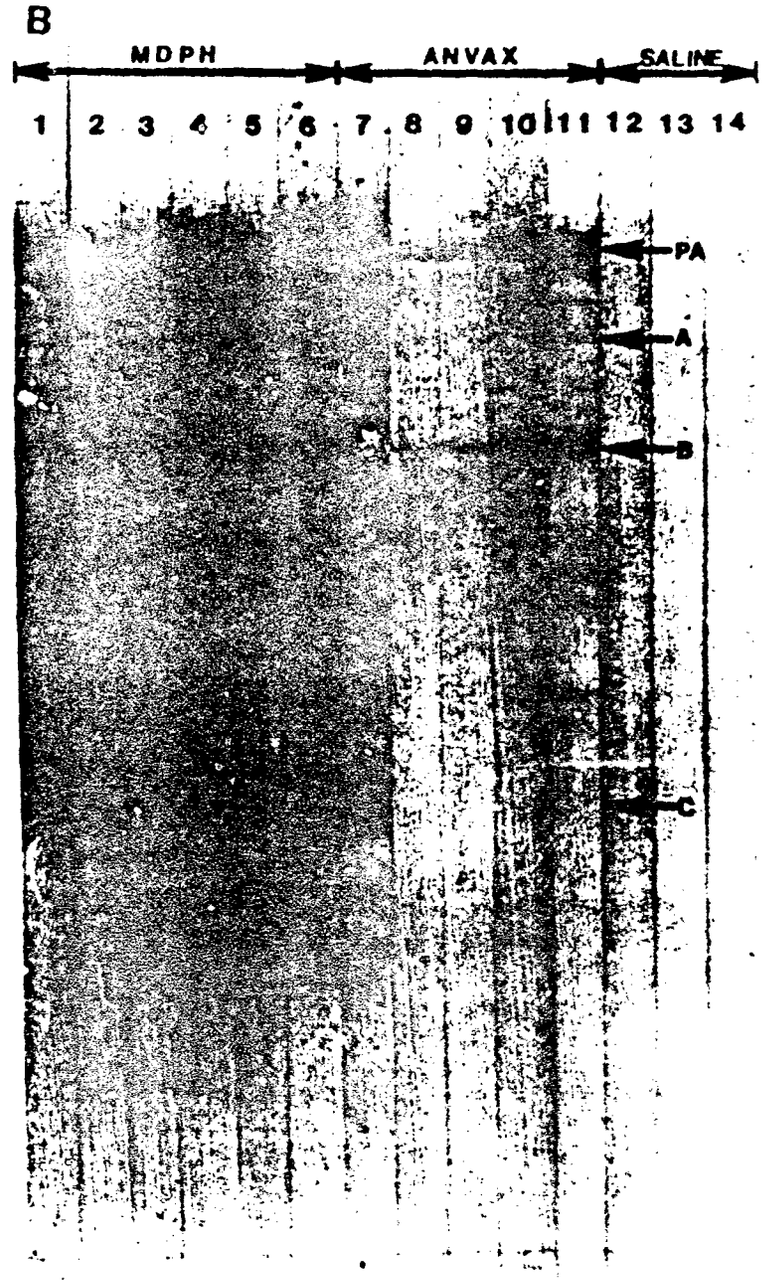
FIG. 2. (A) Comparison of sera from MDPH- and Anvax-vaccinated guinea pigs by EITB on SDS-extracted proteins from Vollum 1-B strain vegetative cells. (B) Comparison of sera by using culture supernatant proteins from the Vollum 1-B cells in Fig. 2-A. The protein preparations were treated as described for FIG. 1. The sera were from animals vaccinated with either the Anvax live spore vaccine (strips 6 to 10) or the MDPH human vaccine (strips 11 to 16). Strips 3 to 5 were incubated with sera from control animals that had been given saline injections. Strips 1 and 2 were reacted with sera from two human subjects that had been vaccinated with 0.5 ml MDPH vaccine (three biweekly injections with booster vaccinations at 6 and 12 months).

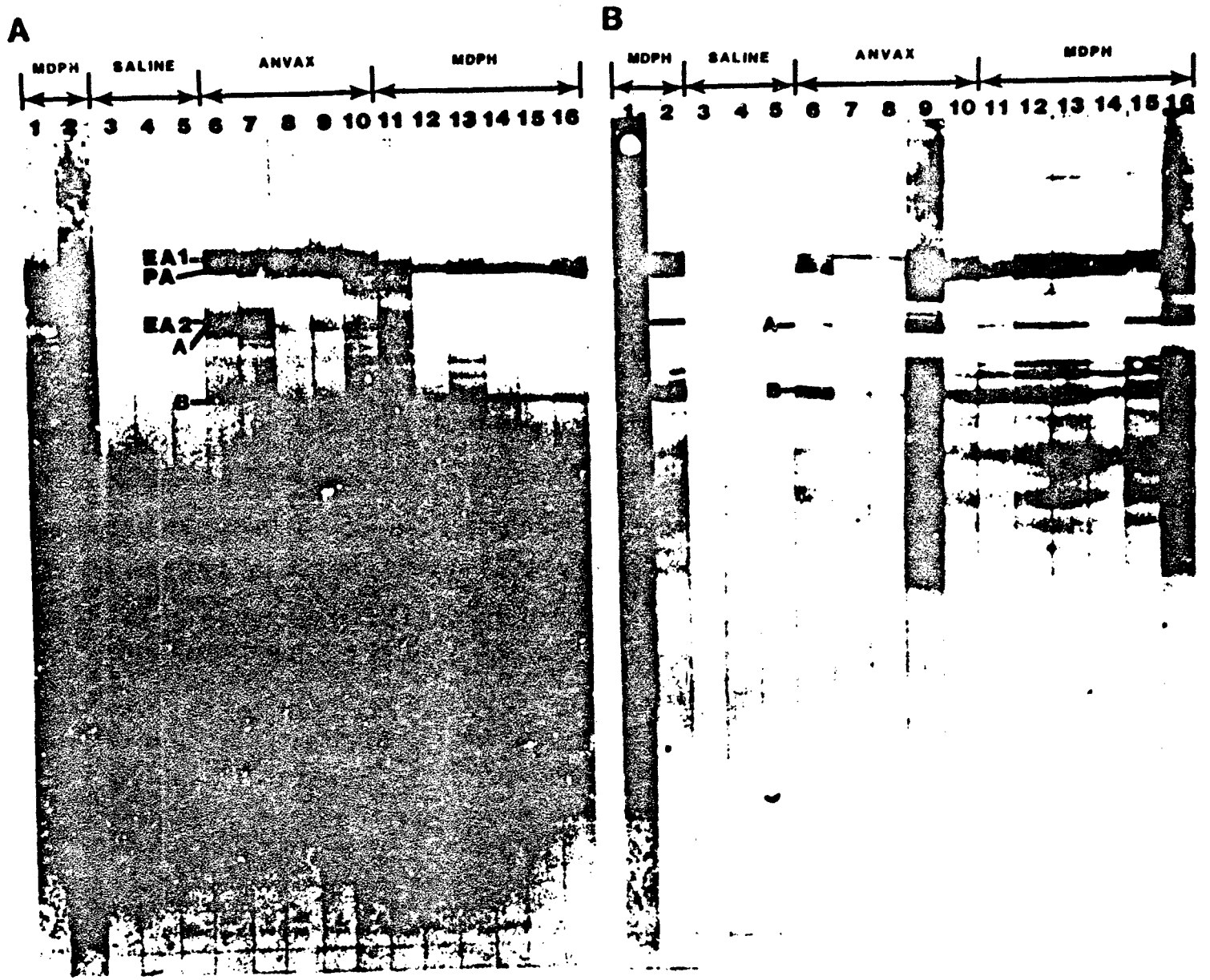
FIG. 3. Approximations of the molecular mass of EA1, EA2, and PA are indicated. Molecular mass protein standards were: (I) ovalbumin, 45 Kdal; (II) bovine serum albumin, 66.2 Kdal; (III) phosphorylase B, 92.5 Kdal; (IV) β-galactosidase, 116.5 Kdal; and (V) myosin, 200 Kdal.

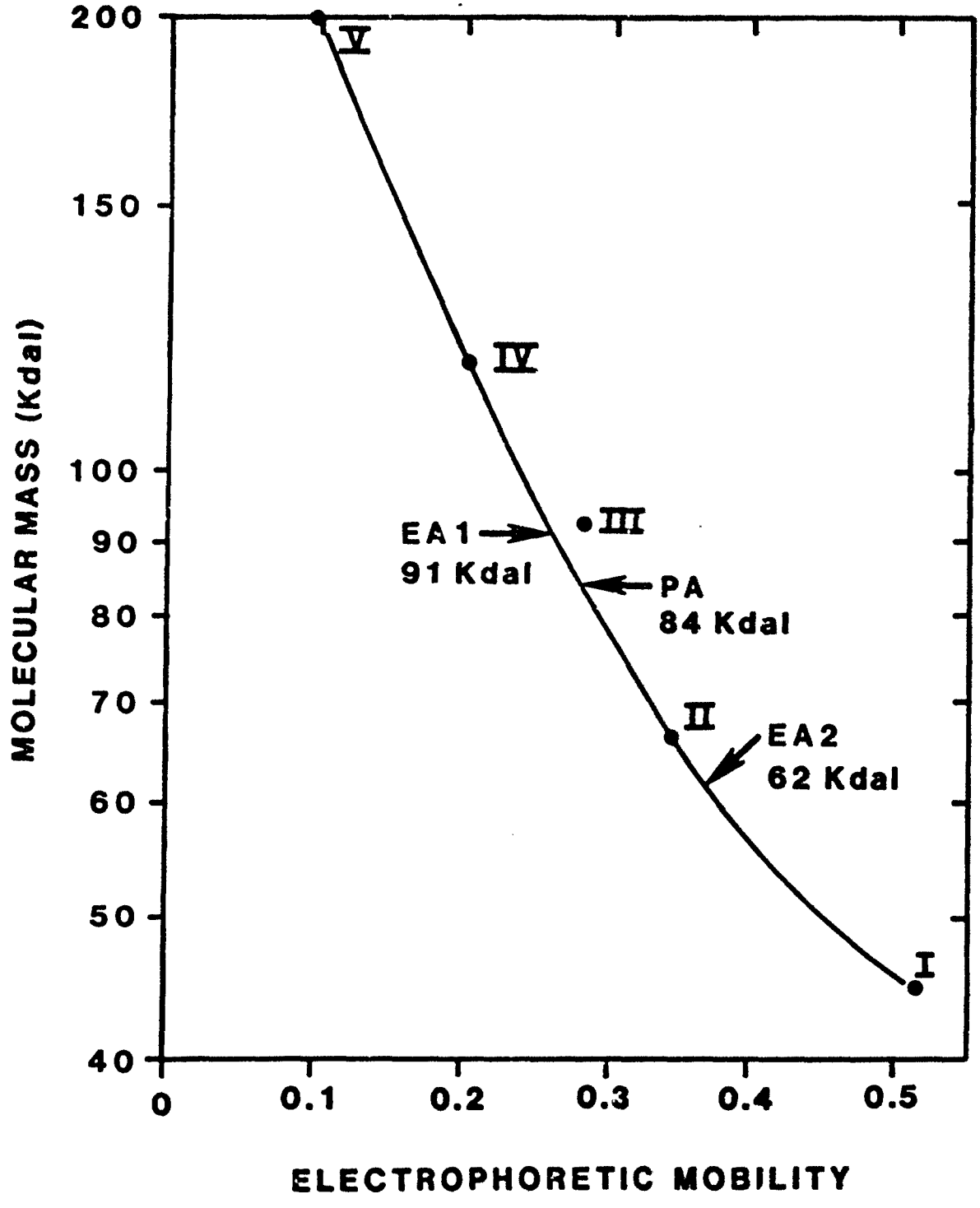
FIG. 4. EITB analysis of Sterne cell-extracted proteins with sera from guinea pigs vaccinated with Anvax (strips 1 and 2) and MDPH (strips 3 and 4). Strips 5 and 6 were reacted with MAb to EF (MAb# EF-III 9F5-1-1, tissue culture fluid) diluted 1:5 and 1:50, respectively. Two different MAb ascities fluid preparations specific for LF (MAb# LF-III 2B2-1-2 and LF-II 3E3-3-1) were used stain strips 7 and 8, respectively. MAb preparations to PA (MAb# PA-I 3B6-1-1 and PA-I 2D3-3-1) were used to stain strips 9 and 10, respectively. The ascities fluids were diluted 1:50.

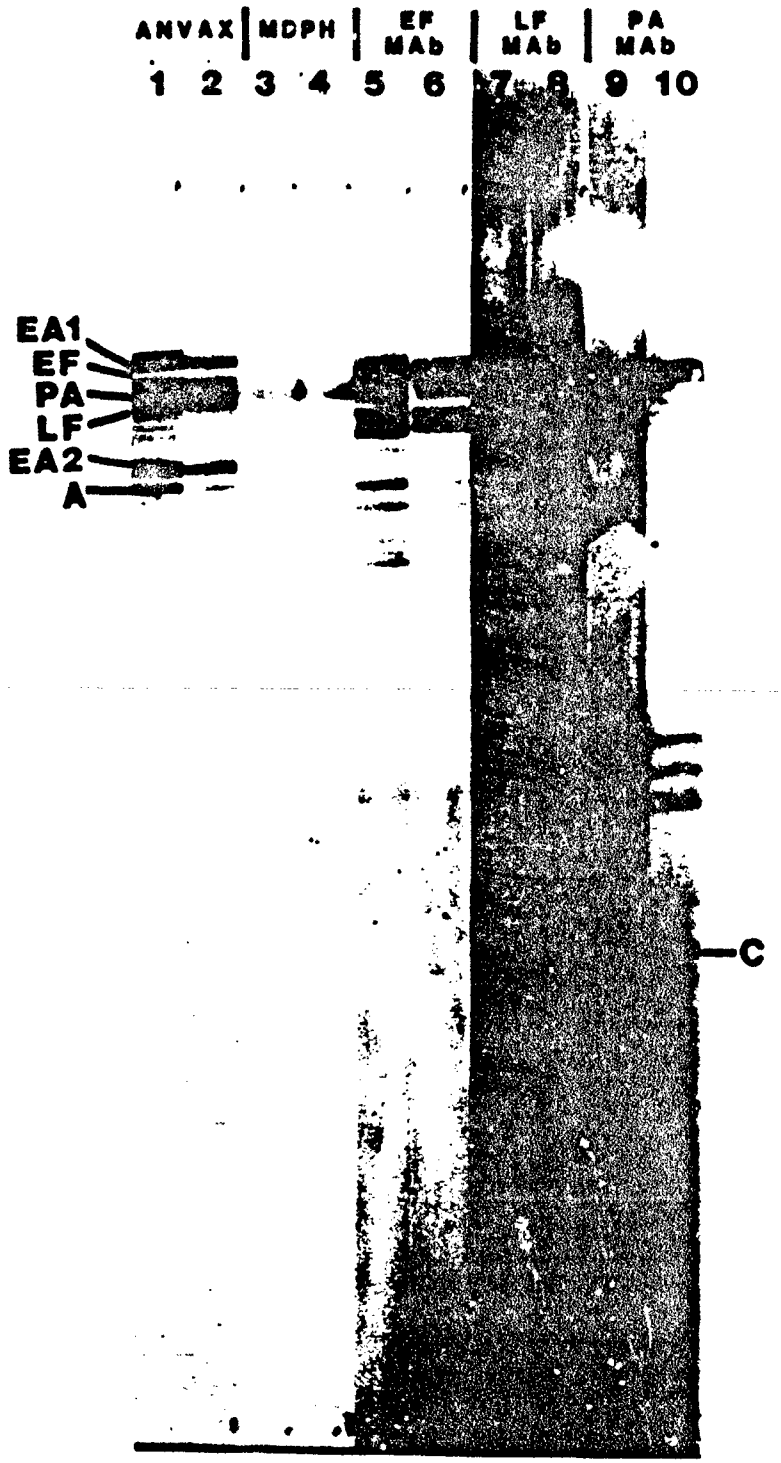
FIG. 5. EITB analysis of Sterne cell extracted proteins with sera from guinea pigs vaccinated with EA1 bound to Nc (strips 1 through 5), Anvax (strips 6 and 7), and MDPH (strips 8 and 9).

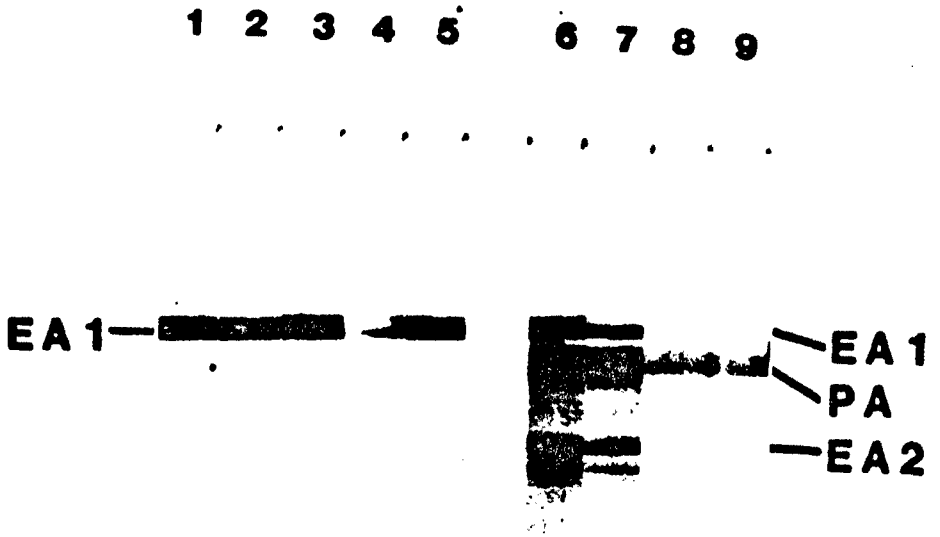

A
MDPH
ANVAX
SALINE
1 2 3 4 5 6 7 8 9 10 11 12 13 14
EA1
PA


B
MDPH
ANVAX
SALINE
1 2 3 4 5 6 7 8 9 10 11 12 13 14
PA
A
B
C


A
MDPH
SALINE
ANVAX
MDPH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
EA1
PA
EA2
A
B
B
MDPH
SALINE
ANVAX
MDPH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
A
B


200
150
100
90
80
70
60
50
40
MOLECULAR MASS (Kdal)
V
IV
III
II
I
EA 1
91 Kdal
PA
84 Kdal
EA 2
62 Kdal
0
0.1
0.2
0.3
0.4
0.5
ELECTROPHORETIC MOBILITY


ANVAX
MDPH
EF MAb
LF MAb
PA MAb
1 2 3 4 5 6 7 8 9 10
EA1
EF
PA
LF
EA2
A
C


1 2 3 4 5 6 7 8 9
EA 1
EA 1
PA
EA 2